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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,421,832**, on March 13, 2003, by **BRADLEY A. SAVILLE AND MIKHAIL I.KHAVKINE,** for "Enhancement of Enzyme Activity by Selective Purification".

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ABSTRACT OF THE DISCLOSURE

A method of enhancing the intrinsic activity of an enzyme in a raw enzyme solution, said method comprising treating said enzyme solution with an effective amount of a purifying agent to effect said enhancement and provide an enzyme solution of enhanced activity.

ENHANCEMENT OF ENZYME ACTIVITY BY SELECTIVE PURIFICATION

FIELD OF THE INVENTION

This invention relates to the use of enzymes for industrial processes, particularly, purification methods advantageous for the enhancement of enzyme activity and stability.

BACKGROUND OF THE INVENTION

The industrial use of enzymes is often limited by their high cost and rapid inactivation. Soluble enzymes are lost with the product at the conclusion of a process, and must be replenished. One area of technological development involves modification of proteins to enhance their activity and/or stability. Processes, such as those involving site-directed mutagenesis and the cultivation of wild forms of enzymes in extreme environments, i.e. extremophiles, have led to significant advances in enzyme technology involving the reduction in the cost per unit of enzyme activity.

Another means to improve the economic feasibility of enzymes for industrial processes is through enzyme immobilization onto a matrix, which may facilitate re-use of the enzyme. Immobilization research has focused upon means to enhance the transfer of enzymes onto the support, and upon means to ensure that the immobilized enzymes remain active. Inactivation of enzymes during catalytic turnover is, however, a key obstacle which may limit the economic feasibility of enzyme-mediated processes. Enzymes may be inactivated by extremes of temperature, pH, shear, and also by free radicals and other reactive species present in the reaction medium. Immobilization technology has the potential to reduce such enzyme inactivation, and, thus, extend the useful lifespan of the enzymes.

Activated carbon is a well-known absorbent and has been previously used for enzyme immobilization via absorption (A.S. Rani, M.L.M. Das, S. Satyanarayana, J. Mol. Catal. B. Enzymatic, 10, 471, 2000), or following derivatization or cross-linking. It is also frequently used for purification of water, beverages, and other process streams. Activated carbon has been used to remove phenolics and phenolic exudates from cultures of A. Canadensis, to facilitate cell growth (G.M. Roy, Activated Carbon Applications in the Food and Pharmaceutical Industries, Technomic Publishing Co.,

Lancaster, PA, 1995). It has also been used for removal of amino acids from protein hydrolysate solutions (Roy, Ibid), and for removal of phenolics from soy protein extracts. Activated carbon has also been used to remove chill-sensitive proteins from beer (J.W. Hassler, Purification With Activated Carbon, Chemical Publishing Co., New York, 1974). However, the prior art is silent as to the effect of activated carbon on the activity of enzyme solutions.

SUMMARY OF THE INVENTION

It is the object of the present invention to produce an enzyme form of enhanced activity for use in industrial processes which improved enzyme form is produced by reagent purification.

Accordingly, in one aspect the invention provides a method of enhancing the intrinsic activity of an enzyme from a raw enzyme solution, said method comprising treating said enzyme solution with an effective amount of a purifying agent, preferably, activated carbon to effect said enhancement and provide an enzyme solution of enhanced activity.

Thus, the invention, as hereinabove defined, results from the surprising discovery that purification of a raw enzyme solution using the purifying agent, most preferably, activated carbon can dramatically enhance the activity of the enzyme solution.

By the term "raw enzyme solution" in this specificaton is meant a commercial grade formulation, produced by fermentation from any one of a variety of bacterial and microbial sources. In the case of an extracellular enzyme, the crude enzyme extract is obtained by, e.g., filtration or centrifugation of the fermentation broth, thus isolating the enzyme from protein debris. If the enzyme is produced intracellularly, the cells are lysed prior to filtration/centrifugation. The crude enzyme extract may also be subjected to membrane separation, ion exchange, or ultrafiltration to produce a partially purified, concentrated enzyme extract rich in the desired enzyme, and relatively devoid of other competing/contaminating enzymes and/or cells. The enzyme solution may also include residual components from the fermentation medium, protease inhibitors, and stabilizing agents.

We have found that the specific enzyme activities, particularly of commercial enzyme formulations are greatly enhanced after purification with, for example, activated carbon.

We have found that the purified enzymes exhibit a significant change in UV-VIS spectra and have increased enzyme activity. Without being bound by theory, we believe that this positive effect of activated carbon purification is a result of improved

enzyme substrate interactions or the removal of inhibitors. Commercial enzyme preparations, formulations and the like, are, generally, colloid solutions that may have a significant amount of dispersed solids, such as, cell debris that may adsorb onto the enzyme and shield the enzyme active centre, and, thus, limit access to bulky substrates, such as starches. Accordingly, enzyme active centre shielding by dispersed solids may, thus, decrease the enzyme specific activity.

Preferably, the enzyme is selected from the group consisting of amylase, glucoamylase, cellulase, xylanase and any other group 3 hydrolase.

The resultant enzyme solution of enhanced activity may be used in admixture with the activated carbon, in its intended subsequent industrial process, such as, the hydrolysis of corn starch, if desired.

Most preferably, the activated carbon is removed, preferably, by filtration or centrifugation, prior to subsequent use of the enhanced activity formulation.

In an alternative method of a practice according to the invention, as herein described, the method comprises a method comprising passing said enzyme solution through a column containing an effective amount of said purifying agent.

Preferably, the raw enzyme solution is diluted with a desired amount of water or aqueous buffer solution for ease of mixing and separation of the activated carbon.

In a further aspect, the invention provides an enzyme formulation of enhanced activity when made by a process as hereinabove defined.

In a further aspect, the invention provides a method of treating a substrate susceptible to enzymatic reaction with an enzyme, said method comprising treating said substrate with an enzyme formulation of enhanced activity as hereinabove defined.

The invention is of particular value in the treatment of polysaccharide products such as, for example, starch from, for example, wheat, potatoes and rice, with alpha-amylase, glucoamylase, cellulase, xylanase, glucose isomerase, or any other group 3 hydrolase.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be better understood, preferred embodiments will now be described by way of example only, wherein

Fig. 1 is a schematic process diagram illustrating a process according to the invention; and

Fig. 2 represents spectral scans of each of (a) raw enzyme, (b) diluted raw enzyme; and (c) purified enzyme.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The following examples illustrate the method according to the invention.

Example 1: Purification of Alpha Amylase with Activated Carbon

A purified enzyme solution was prepared as shown generally as 10 in Fig. 1.

A diluted raw enzyme solution (12), comprising 60 mL raw amylase (Allzyme™, amylase from Alltech) and 270 mL of 0.05M phosphate buffer (pH 6), was prepared and mixed with 24g of activated carbon (14) for 3h with magnetic stirring at 300 rpm in a vessel (16). The purified enzyme (18) was separated from the activated carbon (20) by filtration. Assays of the raw enzyme solution, before dilution (12) and the purified enzyme solution (18) were conducted. The activity of the amylase solution (19) before dilution to produce solution (12) was 2035 U/mL, whereas the activity of the purified enzyme (18) was 2010 U/mL, notwithstanding that, due to dilution, the purified preparation contained only about 18mL of amylase per 100 mL of solution (18). Thus, the activity of the purified enzyme (18), expressed per mL of raw amylase, would be about 11000 U/mL, or about 5.4 times the activity of the original amylase formulation (12). The activity of the diluted enzyme before purification (12) was statistically equivalent to that of the raw enzyme (19), when expressed per mL of raw amylase in the solution.

Example 2: Purification of Alpha Amylase with Activated Carbon

An alternative purified enzyme solution (18) was prepared according to Fig. 1 wherein a diluted raw enzyme solution (12), comprising 40 mL raw amylase (Spezyme™ Fred amylase, from Genencor) and 360 mL of water was prepared and mixed with 8g of activated carbon (14) for 12h with magnetic stirring at 250 rpm in vessel (16). The purified enzyme (18) was separated from the activated carbon (20) by filtration. Assays of the raw enzyme solution before dilution (19) and the purified enzyme solution (18) were conducted. The activity of the amylase solution before dilution (19) was 4486 U/mL, whereas the activity of the purified enzyme (18) was 4170 U/mL, notwithstanding that, due to dilution, the purified formulation (18) contained only about 10mL of raw amylase per 100 mL of solution. Thus, the activity of the purified enzyme (18), expressed per mL of raw amylase, would be about 41700 U/mL, or about 9.3 times the activity of the original amylase formulation (19).

Scheme 1

The aforesaid examples show that purification of these two commercial amylase formulations with activated carbon has led to a clear improvement in activity. As hereinbefore mentioned, this improvement in activity may be due to removal of inhibitors from the enzyme solution, or may be due to removal of dispersed solids, e.g., cell debris that may adsorb onto the enzyme and restrict access of substrates to the enzyme active site. The results show that notwithstanding the significant dilution of the commercial formulations, the purified enzyme solutions according to the present invention possess nearly the same activity as the raw commercial enzyme formulations.

Evidence that treatment with activated carbon has affected the pre-treated enzyme solution is provided through Fig. 2, which shows spectral scans of the raw, undiluted enzyme, the modified enzyme, and the raw enzyme diluted in water. All spectra are normalized with respect to their maximum absorbance values, which are 14.5, 1.0, and 1.43 for the raw, purified, and diluted forms, respectively. Clearly, there is a significant spectral shift. Compared to the raw enzyme solution, the purified preparation exhibits enhanced absorbance in the range from 340 to 380 nm, and a reduction in the absorbance from about 390 to 410 nm. The spectrum for the water-diluted preparation is similar to the spectral profile for the purified enzyme preparation, but exhibits a broader peak from 350 to 360 nm and a depression in absorbance from 390 to 440nm.

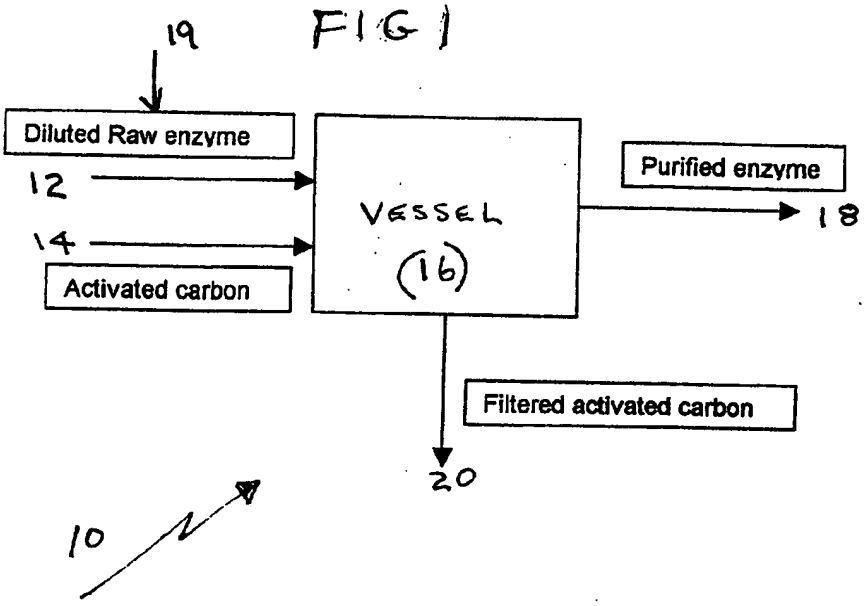
Although this disclosure has described and illustrated certain preferred embodiments of the invention, it is to be understood that the invention is not restricted to those particular embodiments. Rather, the invention includes all embodiments which are functional or mechanical equivalents of the specific embodiments and features that have been described and illustrated.

CLAIMS

1. A method of enhancing the intrinsic activity of an enzyme in a raw enzyme solution, said method comprising treating said enzyme solution with an effective amount of a purifying agent to effect said enhancement and provide an enzyme solution of enhanced activity.
2. A method as defined in claim 1 wherein said purifying agent is activated carbon.
3. A method as defined in claim 1 further comprising removing said purifying agent from said enzyme solution of enhanced activity to provide a purified enzyme solution.
4. A method as defined in claim 1 or claim 2 comprising passing said enzyme solution through a column containing an effective amount of said purifying agent.
5. A method as defined in claim 3 wherein said purifying agent is removed by a method selected from the group consisting of filtration and configuration.
6. A method as defined in any one of claims 1 to 5 wherein said raw enzyme solution is diluted with water to provide a diluted raw enzyme solution.
7. A method as defined in any one of claims 1 to 5 wherein said raw enzyme solution is diluted with an aqueous buffer solution to provide a buffered diluted raw enzyme solution.
8. A method as defined in any one of claims 1 to 7 wherein said enzyme is selected from the group consisting of amylase, glucoamylase, cellulase, xylanase, and any other group 3 hydrolase.
9. A method as defined in any one of claims 1 to 8 wherein said enzyme solution of enhanced activity has a UV-visible spectrum distinct from said raw enzyme solution.
10. A method as defined in any one of claims 1 to 8 wherein said enzyme solution of enhanced activity has a UV-visible spectrum maximum peak at least 30 nm lower than said raw enzyme solution.
11. A method as defined in any one of claims 1 to 10 wherein said enzyme is alpha-amylase and said enzyme solution of enhanced activity has a maximum spectral absorption peak over the range 340 to 360 nm.
12. An enzyme solution of enhanced activity when made by a method as defined in any one of claims 1 to 11.
13. A method of treating a substrate susceptible to enzymatic reaction with an enzyme, said method comprising treating said substrate with an enzyme formulation of enhanced activity as defined in claim 12.

14. A method as defined in claim 13 wherein said substrate is starch and said enzyme is alpha-amylase.

FIG 1



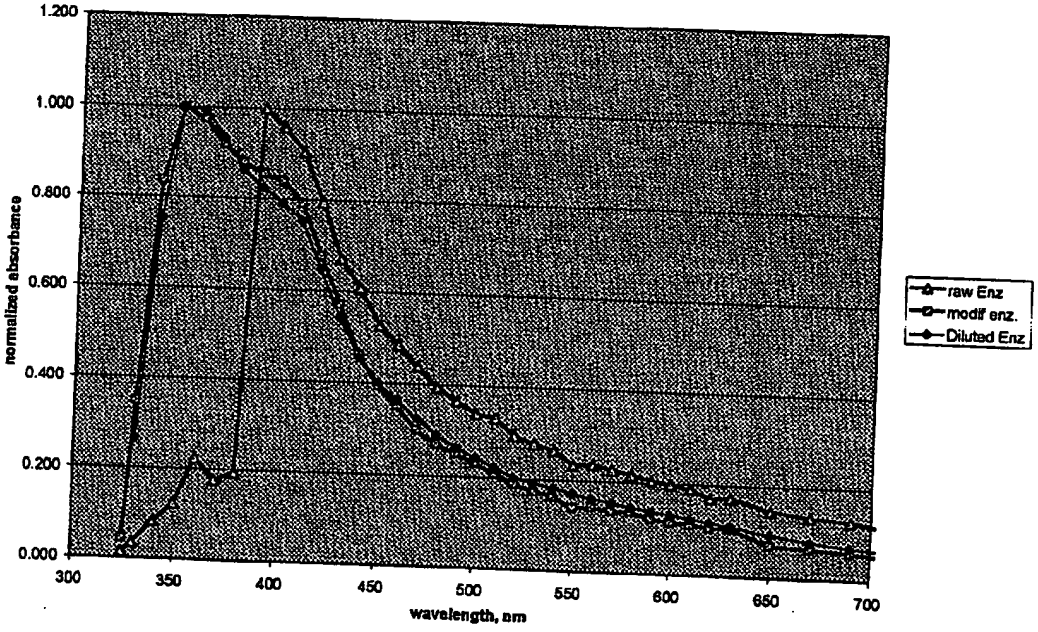


FIG 2